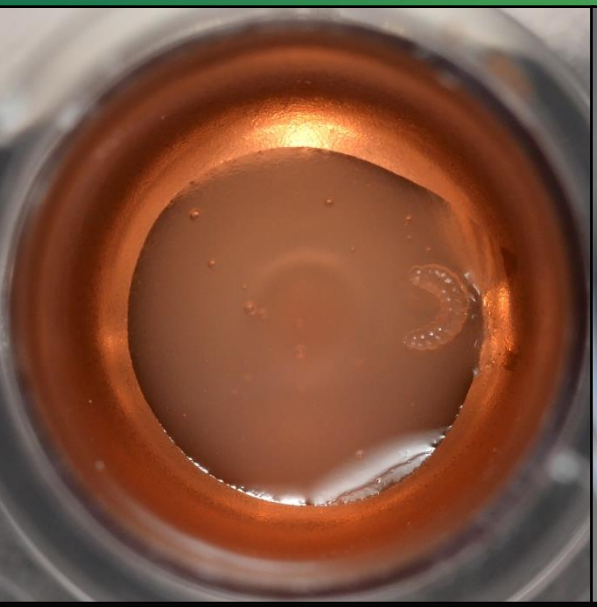
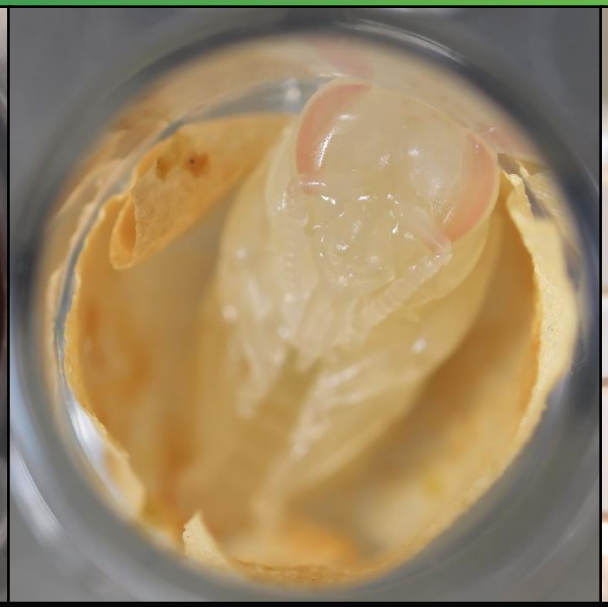
Immature Honey Bee Worker *in vitro* Rearing Workshop

Hosted by the University of Florida, Gainesville, FL
Sponsored by the Pollinator Research Task Force
July 12-14, 2015


Acknowledgements

- University of Florida- facility and transportation
  - Jamie Ellis- Host
  - Brandi Simmons- Coordinator
  - Ashley Mortensen- Preparation, Instruction
  - Mike Bentley- Rearing assay pictures
- CLA Pollinator Research Task Force, DuPont
  - Workshop Sponsors
- Workshop Instructors
  - Dan Schmehl, Bayer CropScience (daniel.schmehl@bayer.com)
  - Ashley Mortensen, University of Florida

Workshop Objectives

1. Understand the immature bee rearing assay protocol using the UF methodology improvements
2. Observe a successful laboratory set-up
3. Design the upcoming *in vitro* immature honey bee ring-test

Workshop Schedule

Monday, July 13th

- 8:15 -8:30 Welcome and overview of workshop objectives (Jamie Ellis, Dan Schmehl)
- 8:30 -10:30 Discussion on critical steps in immature bee *in vitro* rearing assays and identification of the honey bee development stages (Dan Schmehl, Ashley Mortensen)
- 10:30 -10:45 Break
- 1045 -12:00 Guided tour of the UF immature bee *in vitro* rearing assay set-up (Ashley Mortensen, Dan Schmehl)
- 12:00 -13:00 Lunch (on site)
- 13:00 -15:00 Step-by-step *in vitro* rearing methodology discussion (Dan Schmehl, Ashley Mortensen)
- 15:00 – 15:30 Break
- 15:30 – 17:30 Ring-test planning- discussion and limitations (Dan Schmehl)
- 17:30 Transportation back to hotel
- 18:30 Meet in hotel lobby for transportation to dinner (transportation provided)
- 19:00 Group dinner at “Leonardos 706”

Workshop Schedule

Tuesday, July 14th

- 7:30 Pickup at hotel
- 7:45 - 8:15 Breakfast, coffee
- 8:15 - 8:30 Transportation to UF Apiary
- 8:30 - 10:00 Tour of UF Apiary, queen cage and egg laying demonstration (Jamie Ellis, Ashley Mortensen)
- 10:00 - 10:30 Break, transportation to UF laboratory
- 10:30- 12:00 Additional method hands-on training and discussion (Dan Schmehl, Ashley Mortensen)
- 12:00 Transportation to hotel or airport (GNV) as needed

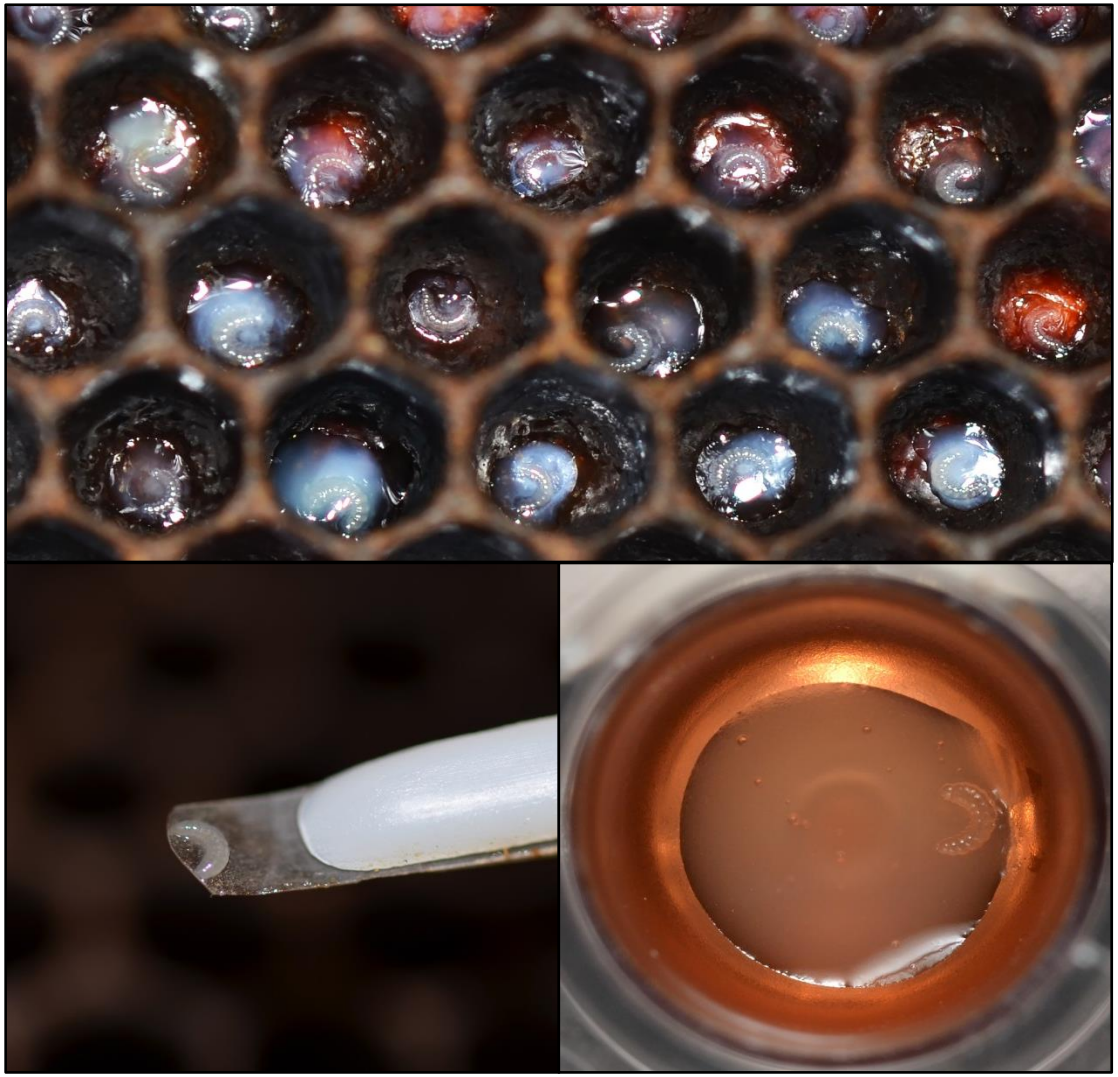
in vitro rearing- UF Improvements

- UF improvements to Aupinel/OCEC methodology
  - Consistent survival over 95% in control and acetone solvent control from time of grafting to adult emergence
- Step-by-step methodology from egg laying to adult emergence
- **Goal: Improve the rate of adult emergence and increase the number of tests satisfying validity criteria**

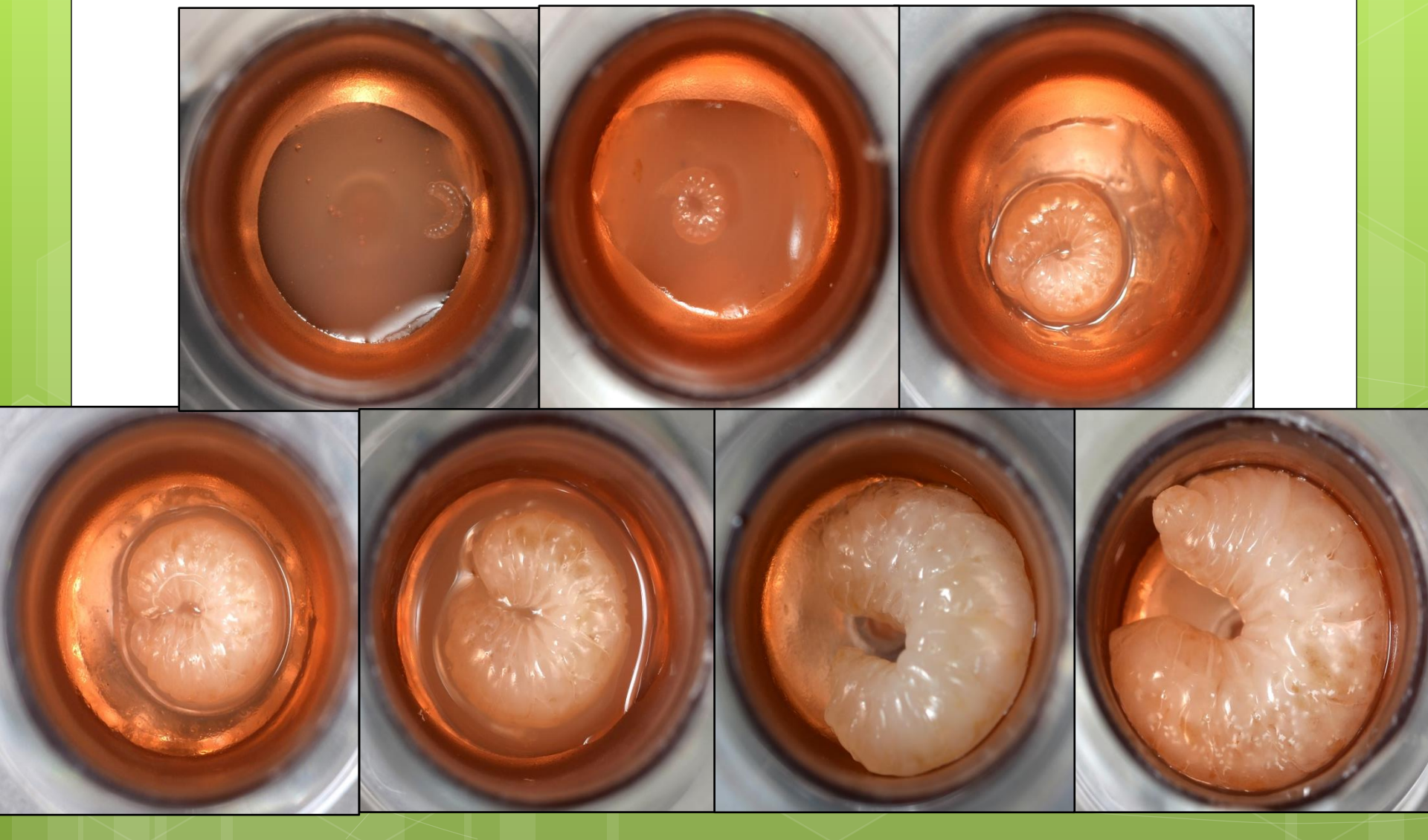
Honey bee *in vitro* development

Critical bioassay steps and identification of the honey bee developmental stages

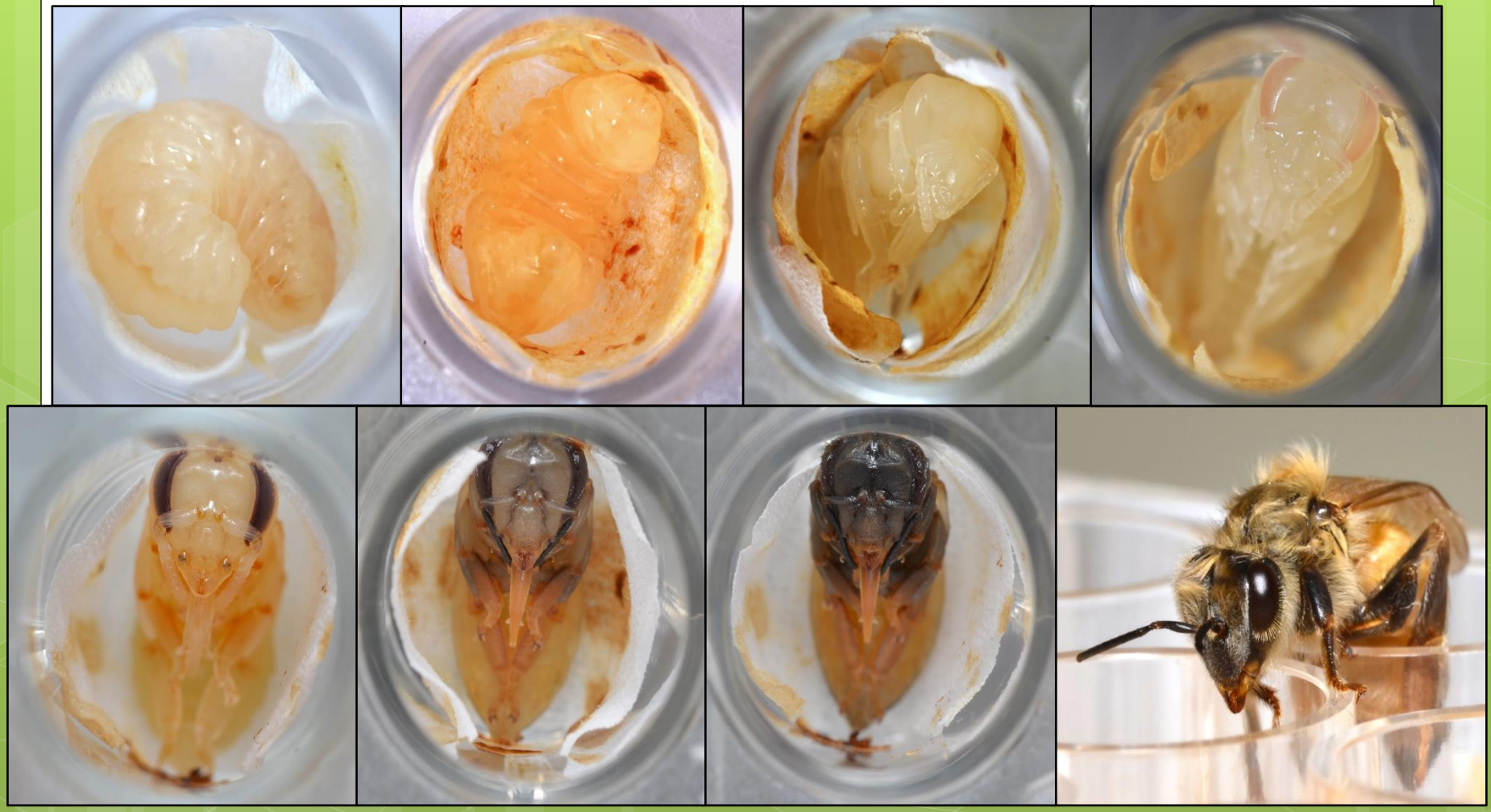
Honey bee *in vitro* development



Honey bee *in vitro* development



Honey bee *in vitro* development



Honey bee *in vitro* development

OECD Guidance, Repeated Exposure

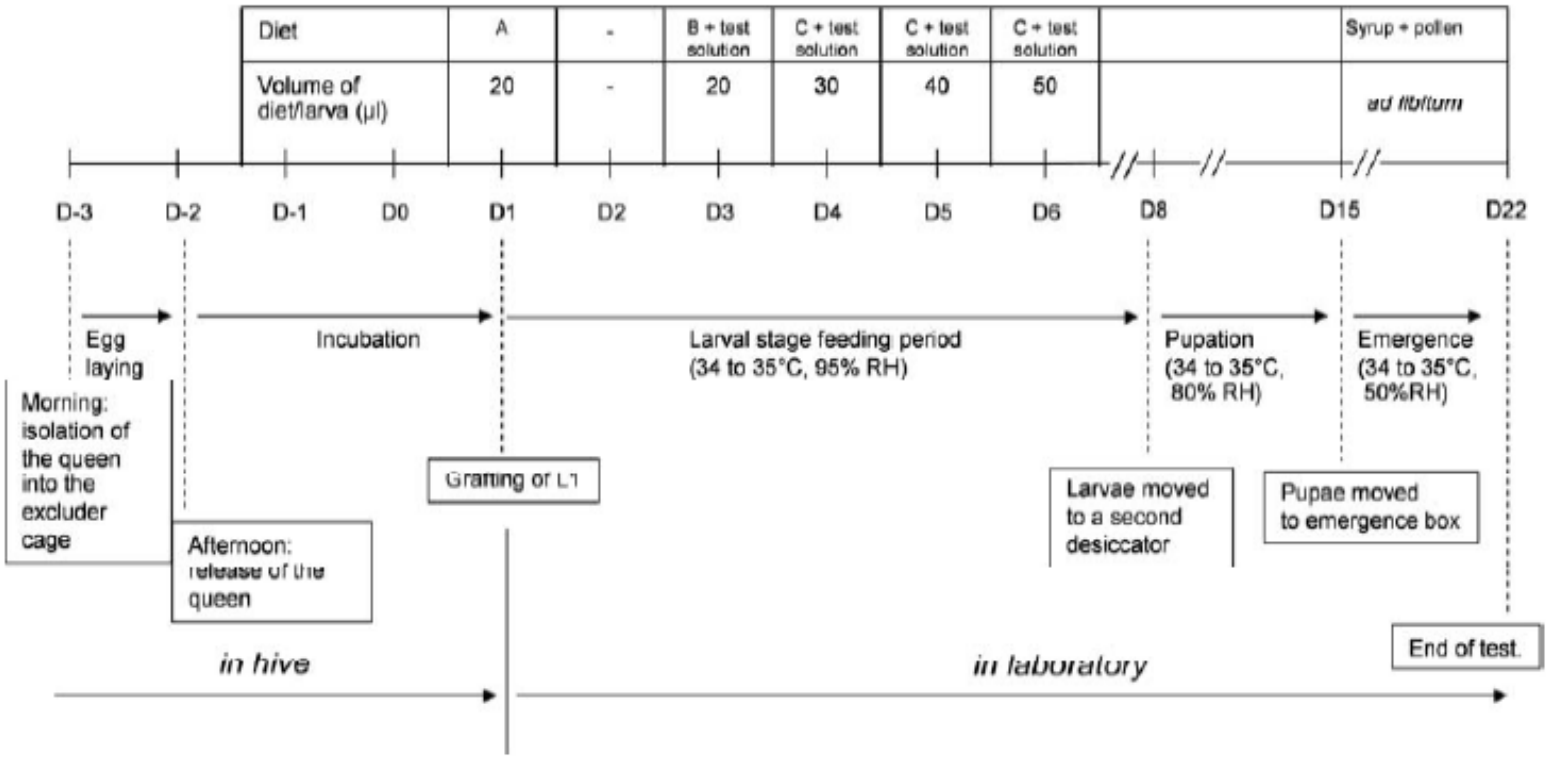


Figure 5: Schematic representation of the important steps of the larval repeated exposure toxicity test (D = day; RH = relative humidity)

Critical Take Home Message

- Sterile Environment/Technique
- Diet
  - Royal Jelly Source
  - Proportion of Ingredients- More H_2O, Less RJ
- Larvae/Pupae Transfer Time
- Many Unknowns
  - Seasonality
  - Region Variation
  - Colony Variation
  - Diet
  - Biological Function

in vitro Rearing Supplies

Table 1. Tools and Supplies Needed

	Item letter (corresponds to table references in the text)	Item and Description	Quantity (minimum)
Equipment	A	Heratherm incubator (Thermo Scientific, #IMH750-S), or equivalent. The incubator must maintain temperature of ± 0.5°C.	1 unit
	B	Freezer (-20°C)	1 unit
	C	Refrigerator (+4°C)	1 unit
	D	Clean hood, positive pressure (air flow set at 0.5 inches of water)	1 unit
	E	Microwave, 1000 watt	1 unit
	F	Space heater, 1500 watt ceramic (Comfort Zone, #C2442WN)	1 unit
	G	30.5 cm^3 desiccators (Thermo Scientific, #08-642-21)	2 units
	H	Data loggers (Onset, HOBO #UX100-011)	2 units
Queen caging and transportation	I	Honey bee colonies (Langstroth hive)	3 units
	J	Zinc queen excluder push-in cage (10 cm × 10 cm × 3 cm)	4 cages
	K	ThermiPaq heat packs, clay based, 15 cm × 30 cm (Medical Supply 123, #201)	2 packs
	L	Five-frame honey bee hive box with two telescoping outer covers	1 box with two

in vitro Rearing Supplies

Larval diet preparation	M	D-fructose (Fisher, #L95-500), store at room temperature	1 container
	N	D-glucose (Fisher, #D16-500), store at room temperature	1 container
	O	Bacto yeast extract (Bacto, #288620), store at room temperature	1 container
	P	Royal jelly (Stakich). The source of royal jelly is of very important and it should be vetted appropriately (see Discussion-'Larval Diet Composition' for more details) prior to use. Stakich brand royal jelly has been used successfully and reliably for rearing larvae. All royal jelly should be shipped via overnight delivery. It should be stored at -20°C upon arrival.	1 container
	Q	ddH_2O or autoclaved water	1 gallon
	R	Stainless steel laboratory spatula (Fisherbrand, # 14-373)	3 tools
	S	Stainless steel laboratory scoopula (Fisherbrand, #14357Q)	3 tools
	T	100 mL glass beakers (Corning Life Sciences, #07-250-054), or equivalent	3 beakers
Honey bee grafting and maintenance	U	Chinese grafting tools (GloryBee, #14513) or similar grafting tool. The grafting tools should be made of plastic or metal so that they can be sterilized easily. Wooden grafting tools are not acceptable.	10 tools
	V	48-well tissue culture plates, sterile (Falcon, #353230) or equivalent. Plates wil be prepared in two different ways for larval rearing. The larvae will be reared in larval sterile tissue culture plates (STCP), while the pupae will be transferred to and maintained in pupal STCP.	1 case
	W	Brown plastic cell cups (GloryBee, #14332). The cell cups need to fit within the STCP wells, therefore other cell cup designs may not work for rearing protocol.	10 bags
	X	Pipette, variable volume 10-100 µL (Sigma-Aldrich, #Z683809)	1 unit

in vitro Rearing Supplies

	X	Pipette, variable volume 10-100 µL (Sigma-Aldrich, #Z683809)	1 unit
	Y	Pipette tips, sterile filtered 1-200 µL (Sigma-Aldrich, #CL54823)	1 box
	Z	Kimwipes (Kimberly Clark, #06-666A)	1 box
	AA	Sodium Chloride (NaCl, Sigma Aldrich, #S7653-1Kg)	1 container
	BB	Potassium Sulfate (K_2SO_4, Sigma Aldrich, #223492-2.5Kg)	1 container
	CC	Fiber optic light source, or equivalent	1 unit
Sterilization	DD	Face mask (Global Industries, #T9F954219)	2 masks
	EE	UV light	1 light
	FF	Nitrile gloves (Fisher Scientific, #19-167-051)	1 box
	GG	Aluminum foil	1 box
	HH	Bleach (10% v/v)	1 container

* Supplies may vary in style or manufacturer

** Items not included in list: 0.22um filter

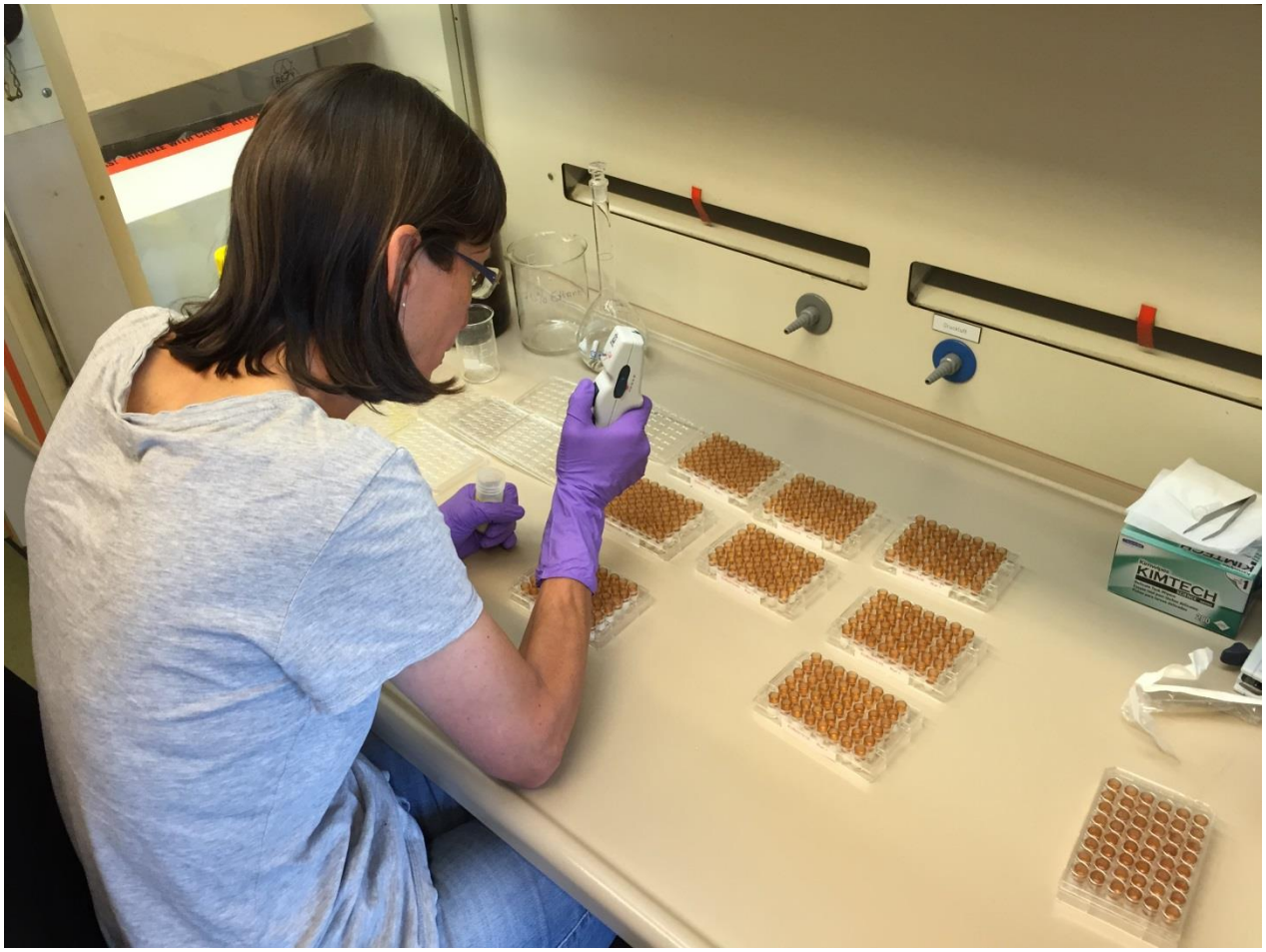
in vitro Rearing- *Sterile Environment*

Tools

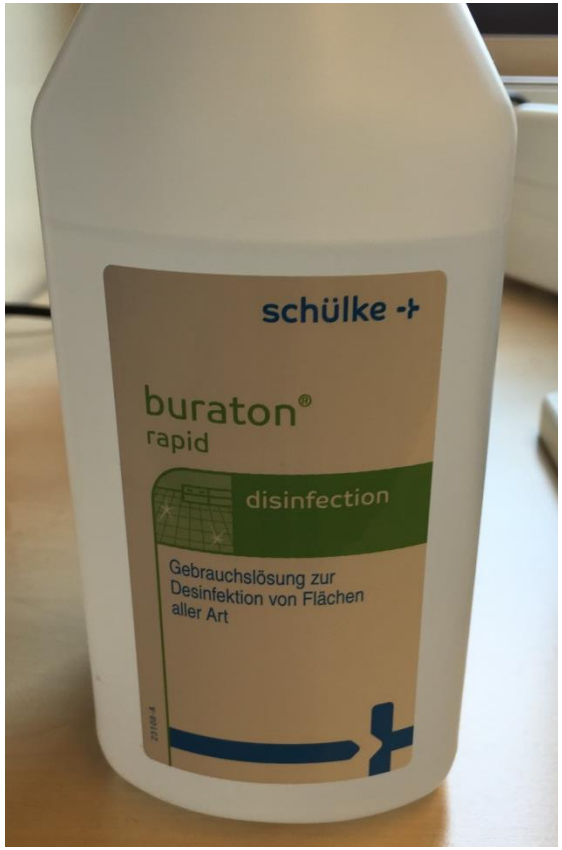
Grafting Environment

Rearing Environment

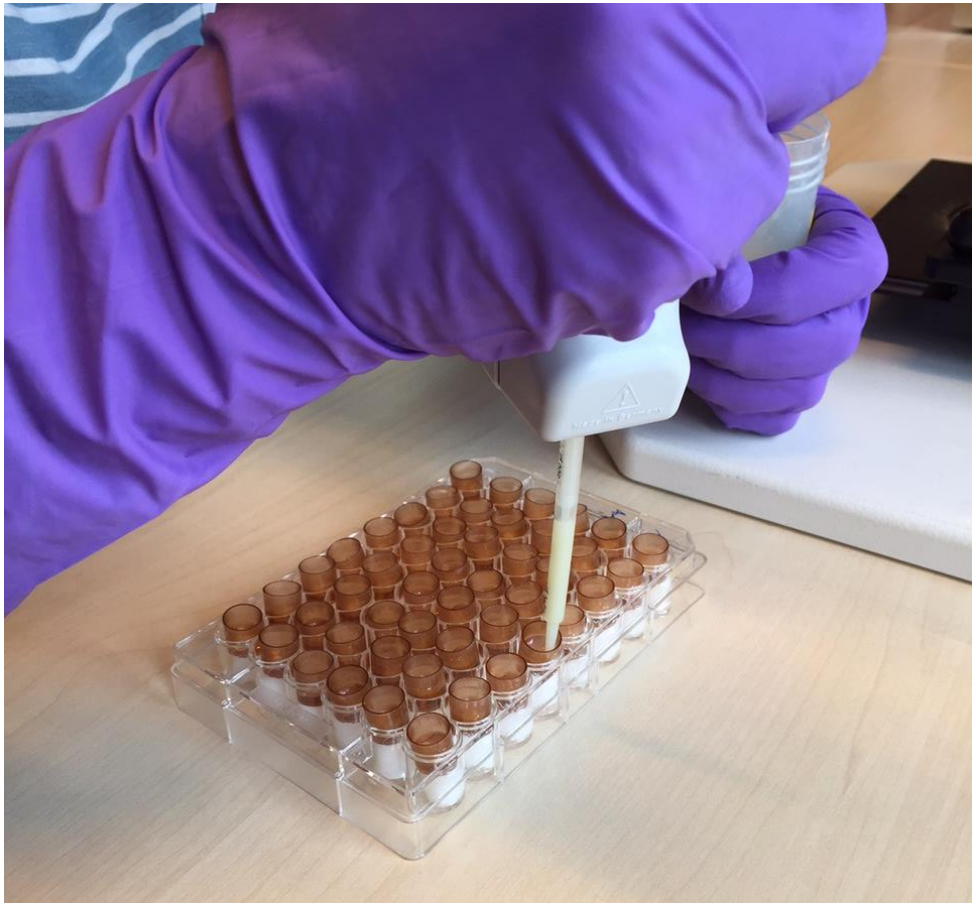
in vitro Rearing- Good *Technique*



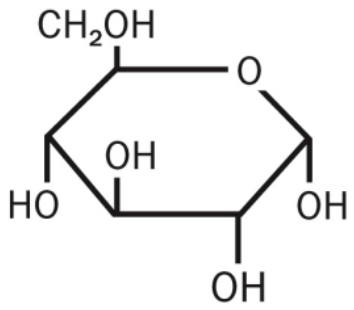
in vitro Rearing- Good *Technique*

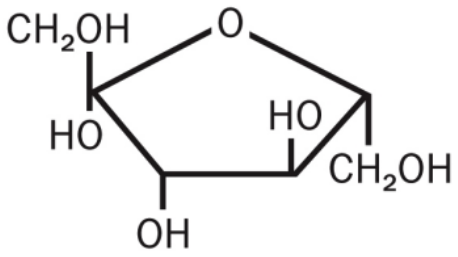


in vitro Rearing- Good *Technique*



in vitro Rearing-Diet Composition





in vitro Rearing- Diet Composition

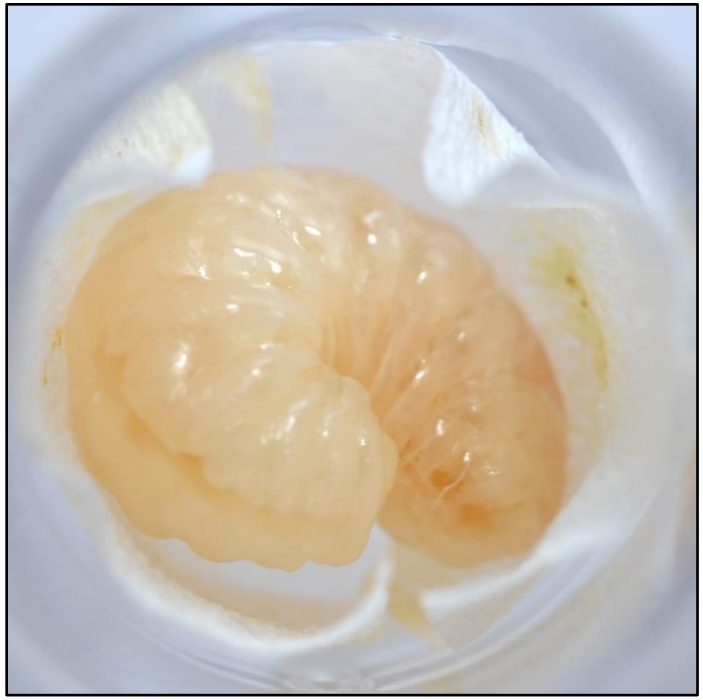
	AMOUNT OF LARVAL DIET COMPONENTS (~10g diet)					
	OECD/OECE 237			UF Method		
	5 g RJ + 5g aqueous solution			10 g total weight		
	Diet A	Diet B	Diet C	Diet A	Diet B	Diet C
Royal Jelly (g)	5.00	5.00	5.00	4.43	4.30	5.00
Glucose (g)	0.60	0.75	0.90	0.53	0.64	0.90
Fructose (g)	0.60	0.75	0.90	0.53	0.64	0.90
Yeast Extract (g)	0.10	0.15	0.20	0.09	0.13	0.20
Water (g)	3.70	3.35	3.00	4.43	4.30	3.00

in vitro Rearing- Diet Composition

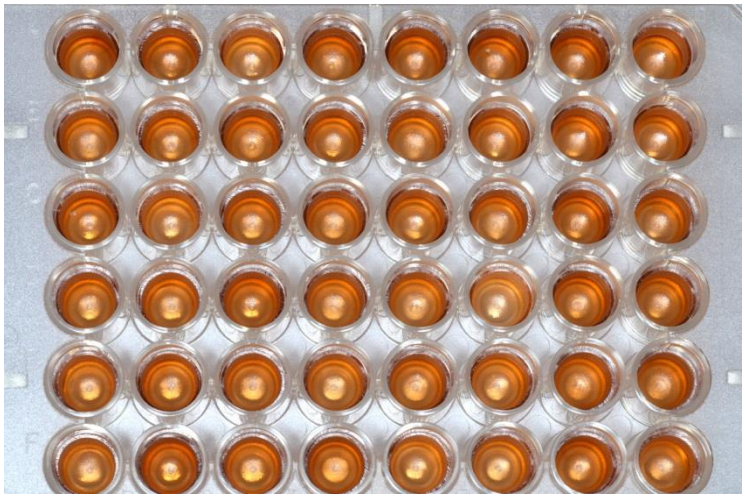
Table 2. Amount and percentage of diet components in the larval diet for ~400 larvae

Diet component	Amount of diet components			Percentage of diet components in total diet		
	Diet A	Diet B	Diet C	Diet A	Diet B	Diet C
Royal Jelly (g)	4.43	4.30	25.00	44.25%	42.95%	50.00%
Glucose (g)	0.53	0.64	4.50	5.30%	6.40%	9.00%
Fructose (g)	0.53	0.64	4.50	5.30%	6.40%	9.00%
Yeast Extract (g)	0.09	0.13	1.00	0.90%	1.30%	2.00%
Water (g)	4.43	4.30	15.00	44.25%	42.95%	30.00%
Total	10 g	10 g	50 g	100%	100%	100%

in vitro Rearing- Pupal Transfer



in vitro Rearing- Larval Plate Conditions



- UV sterilized (plate, tools)
- No glycerol solution used
- Filtered (0.22µm) H_2O
- No antibiotics added
- Lid present
- Gap between cup and lid

Honey bee *in vitro* development

Step-by-step *in vitro* rearing methodology

Methodology- Grafting Timeline

Table 3. In vitro rearing time reference points. For the "age of bee from t = 0," 0 is the midpoint of the time the queen was caged. Once the queen is released, the eggs she laid are 0 ± 12 h old if she was caged for 24 hours. We discuss the tasks performed at each time point in the "Task performed" column. We also provide a sample time schedule that aligns with the mentioned tasks and puts all tasks associated with the rearing protocol at reasonable times of the day.

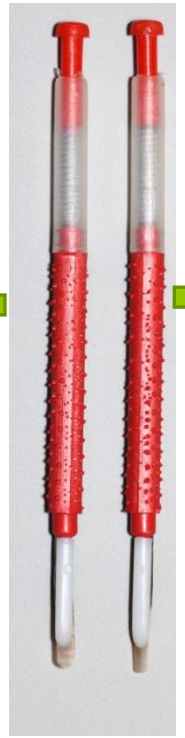
Time d (h) since initiating in vitro rearing protocol	Time d (h) recognizing grafting as t=0	Age of bee d (h) from t = 0. All times are ± 0.5 d or 12 h.	Task performed	Sample daily time schedule
-0.5 (-12)	-4 (-99)	-0.5 (-12)	Cage queen	10:00
1 (24)	-3 (-75)	0.5 (12)	Release queen	10:00
2 (48)	-2 (-51)	1.5 (36)	N/A	
3 (72)	-1 (-27)	2.5 (60)	N/A	
4 (99)	0	3.625 (87)	Graft /Feeding	13:00
5 (123)	1 (24)	4.625 (111)	Inspection	13:00
6 (147)	2 (48)	5.625 (135)	Feeding/Inspection	13:00
7 (171)	3 (72)	6.625 (159)	Feeding/Inspection	13:00
8 (195)	4 (96)	7.625 (183)	Feeding/Inspection	13:00
9 (219)	5 (120)	8.625 (207)	Feeding/Inspection	13:00
10 (243)	6 (144)	9.625 (231)	Pupal Transfer/Inspection	13:00

Methodology- Grafting Timeline

11 (267)	7 (168)	10.625 (255)	Pupal Transfer/Inspection	13:00
12 (2918)	8 (192)	11.625 (279)	Pupal Transfer/Inspection	13:00
13 (315)	9 (216)	12.625 (303)	Inspection	13:00
14 (339)	10 (240)	13.625 (327)	Inspection	13:00
15 (363)	11 (264)	14.625 (351)	Inspection	13:00
16 (387)	12 (288)	15.625 (375)	Inspection	13:00
17 (411)	13 (312)	16.625 (399)	Inspection	13:00
18 (435)	14 (336)	17.625 (423)	Inspection	13:00
19 (459)	15 (360)	18.625 (447)	Inspection	13:00
20 (483)	16 (384)	19.625 (471)	Inspection	13:00
21 (507)	17 (408)	20.625 (495)	Inspection	13:00
22 (531)	18 (432)	21.625 (519)	Inspection/Adult Emergence	13:00
23 (555)	19 (456)	22.625 (543)	Inspection/Adult Emergence	13:00
24 (579)	20 (480)	23.625 (567)	Inspection/Adult Emergence	13:00
25 (603)	21 (504)	24.625 (591)	Inspection/Adult Emergence	13:00
26 (627)	22 (528)	25.625 (615)	Inspection/Adult Emergence	13:00

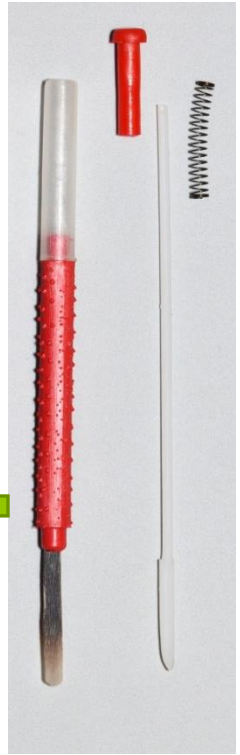
Methodology- Prepping Tools

- Chinese Grafting Tool
- Prepupal Transfer Tool



Original

Modified for grafting



Disassembled grafting tool for prepupal transfer

Methodology- Grafting Tool Options

Modified Chinese Grafting Tool

Dental Tool

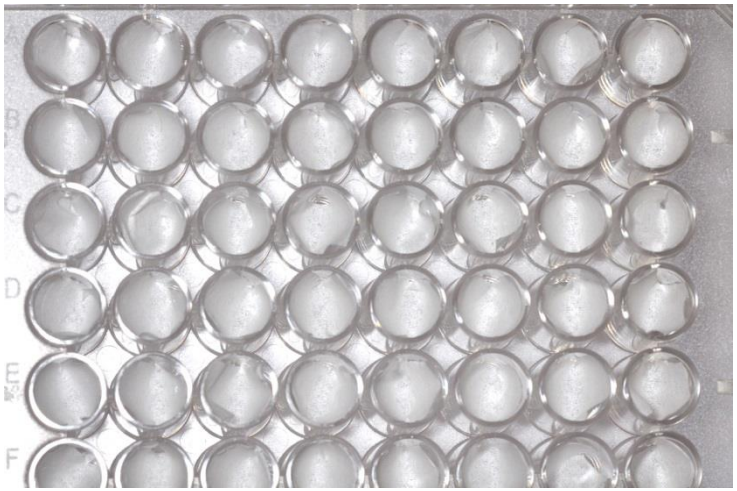
00 Camelhair Paintbrush

Methodology- Plate Set-up

- Larval STCP

Queen cups placed in each cell
No sterilizing solution

- Pupal STCP



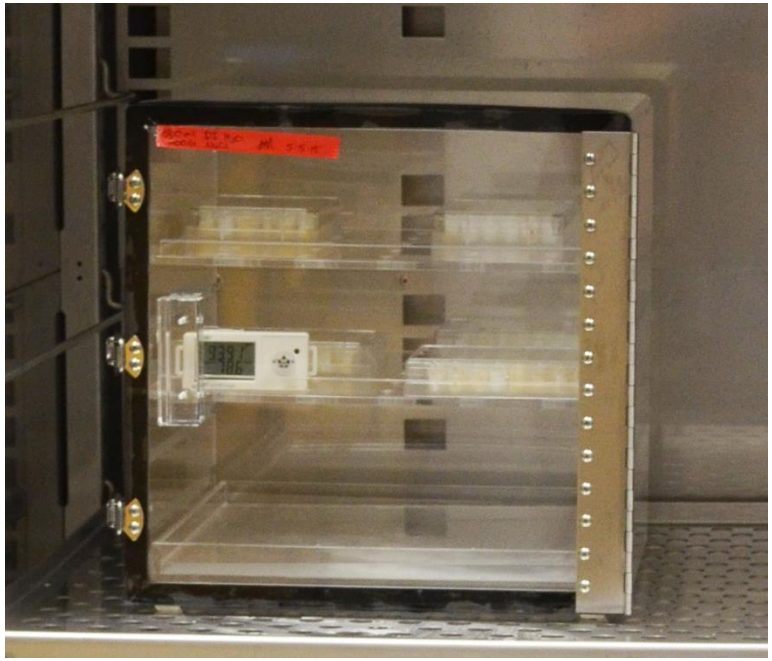
Kimwipe placed within each cell

Methodology- Sterilize tools

Hood equipped with UV light,
Models Vary- Use according to manufacturer's instructions

Methodology- Clean Workspace, Salt Solutions

Clean bench space, hood, incubator, and desiccators with 10% bleach solution



Larval chamber- 94% R.H.
-160 mg K_2SO_4 to 1L H_2O

Larval chamber- 75% R.H.
-320 mg NaCl to 1 L H_2O

*Replace salt solutions weekly

Methodology- Incubator

Warm Incubator to 35°C

No need to have humidity control

Methodology- Bench top/hood

Keep workspace warm (~31°C)
- **Space heater (if using hood)**
- **Coil or hot plate**

***Use the UF suggested diet to prevent diet from drying out during grafting and maintenance**

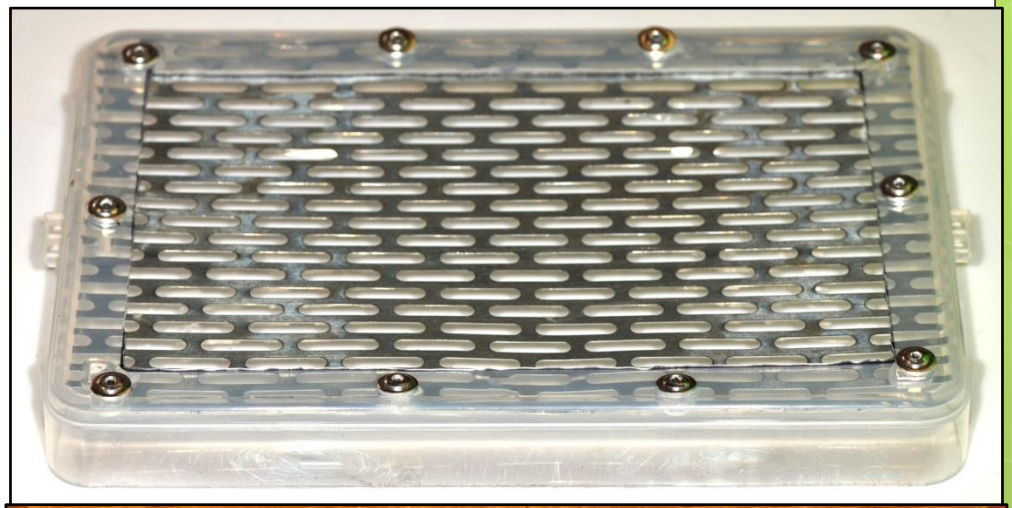
Methodology- Larval Diet

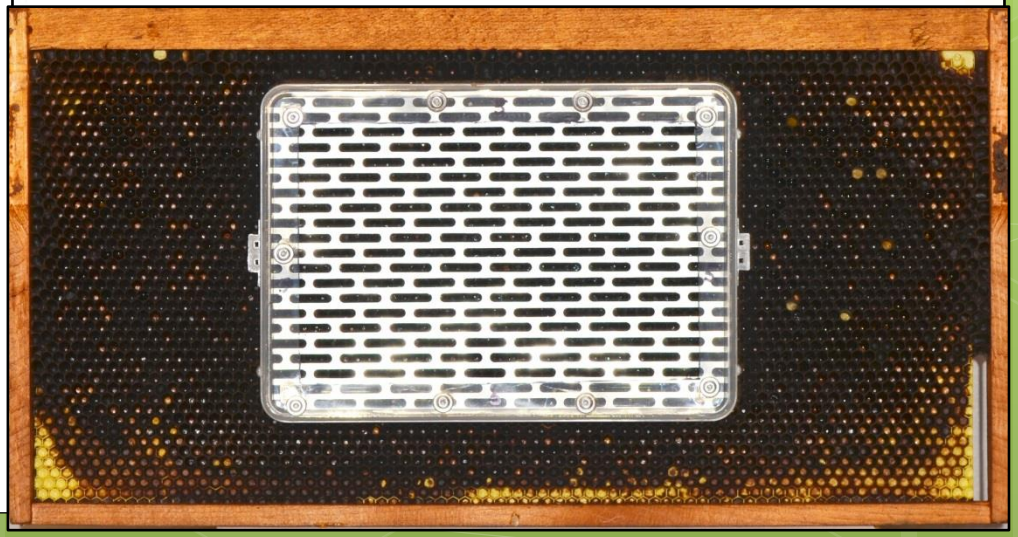
Mix diet in the following order:
-Measure the amount of **ddH_2O** (room temperature) needed.
-Add the two sugars, **D-fructose** and **D-glucose**, to the water and mix with a spatula until the sugars dissolve completely.
-Add the **yeast** and mix all ingredients until completely dissolved.
-Add the **royal jelly** and mix until it is completely dissolved.

Three different diets
-Diet A- Day 0
-Diet B- Day 2
-Diet C- Day 3,4,5

***Reference earlier charts for ingredient amount/proportion**

Methodology- Collecting Larvae





Cage Queen using queen excluder material (designs will vary)

Release queen after 24 hours (keep eggs within excluder)

Methodology- Collecting Larvae

Collect young larvae 75 hours after queen is released from cage.

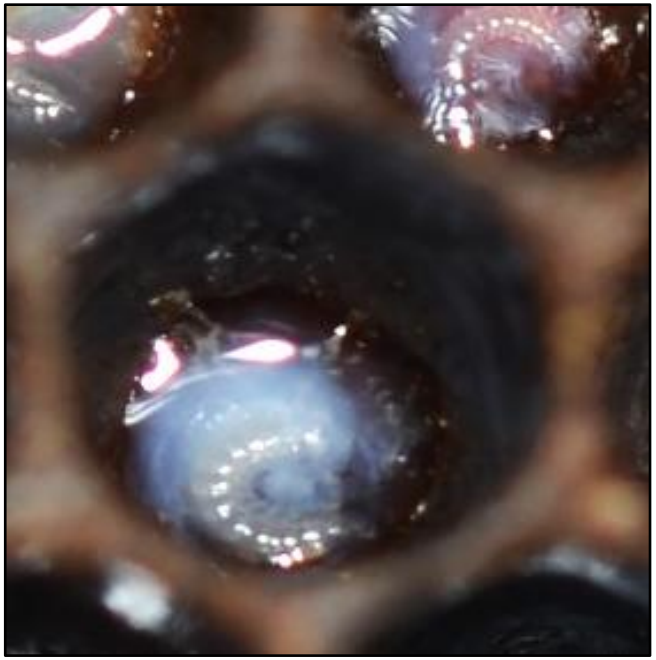
The age of the larvae is 87 ± 12 hrs.

Methodology- Transporting Larvae to Lab

Transport larvae to the lab in an insulated box equipped with a heat source

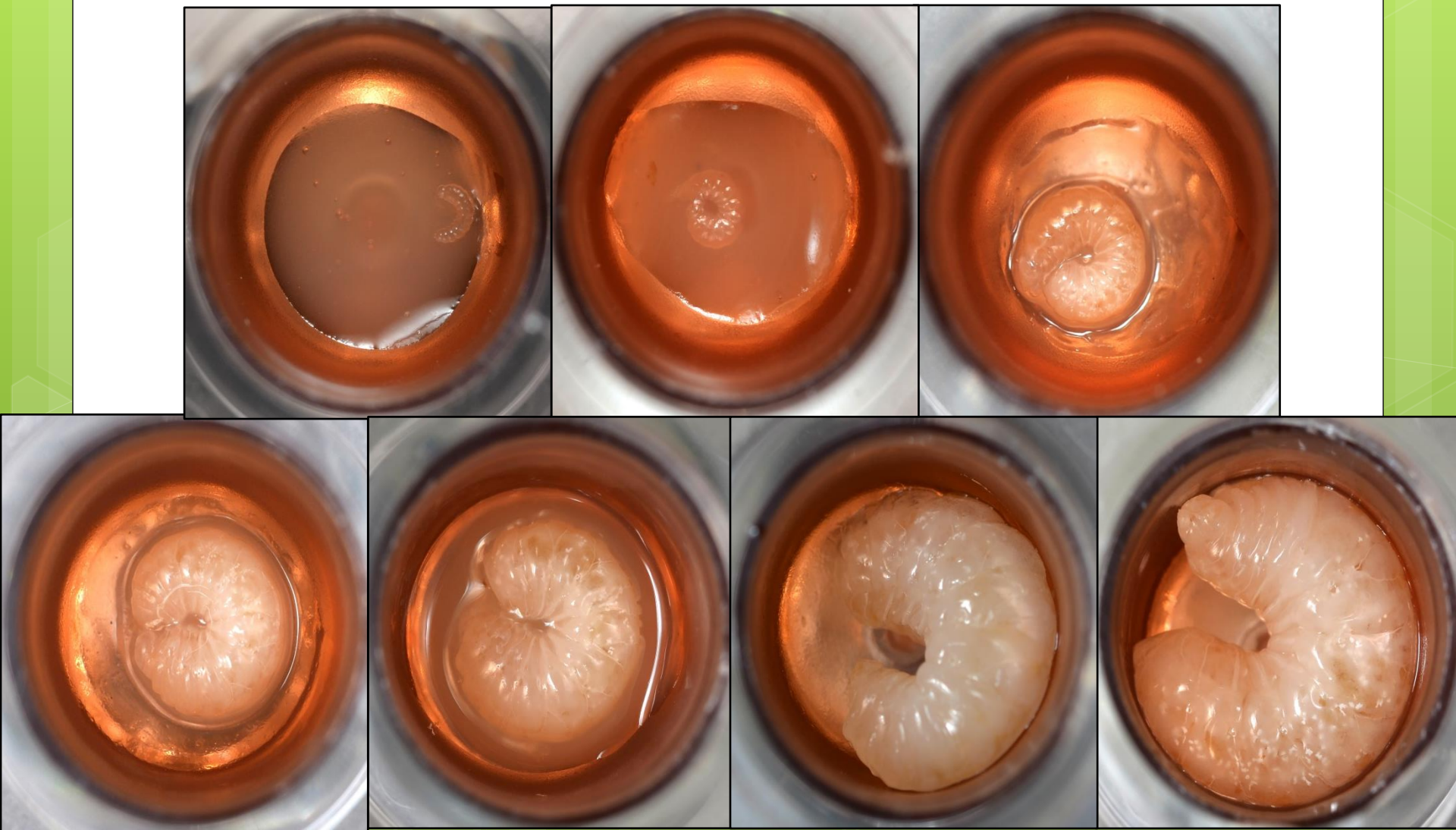
Once frames arrive in the lab, keep frames at hive temperature until grafting commences.

Methodology- Grafting Larvae

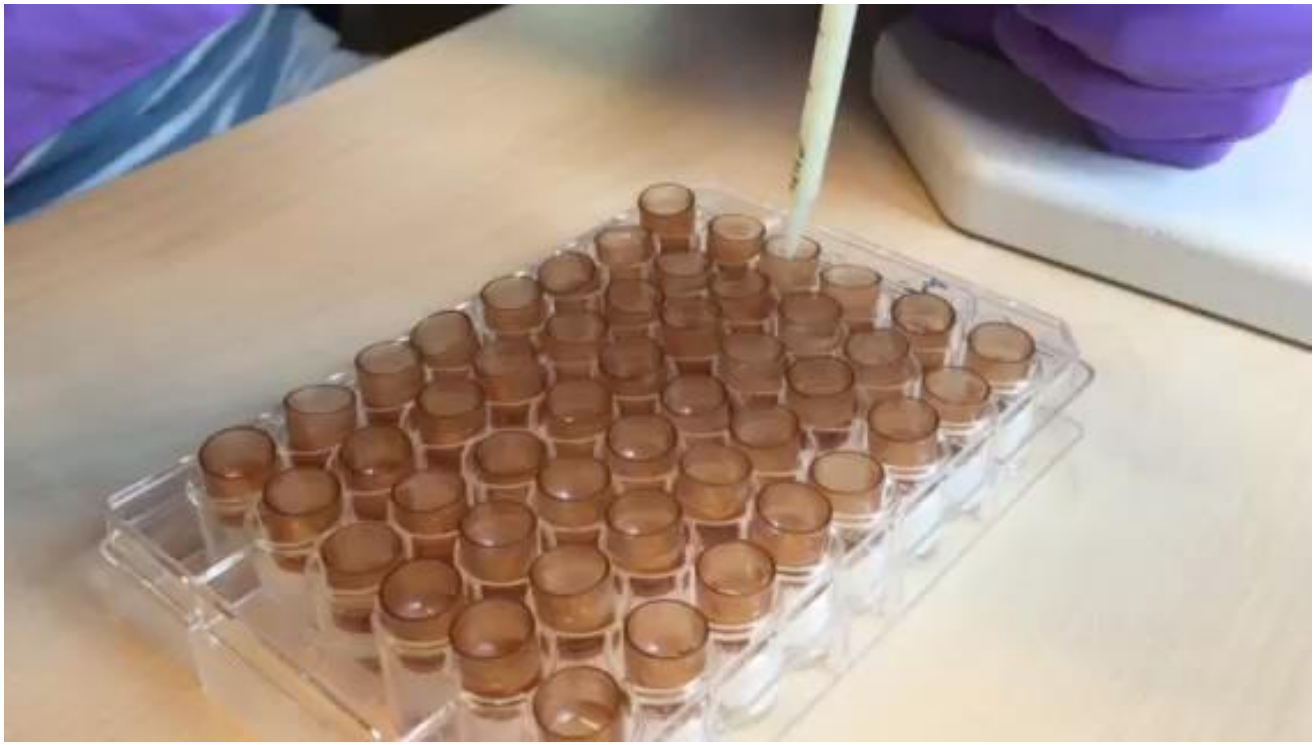


Transfer larvae to larval STCP using a grafting tool that you are comfortable using and results in high larval survival

Methodology- Feeding Larvae



Methodology- Feeding Larvae



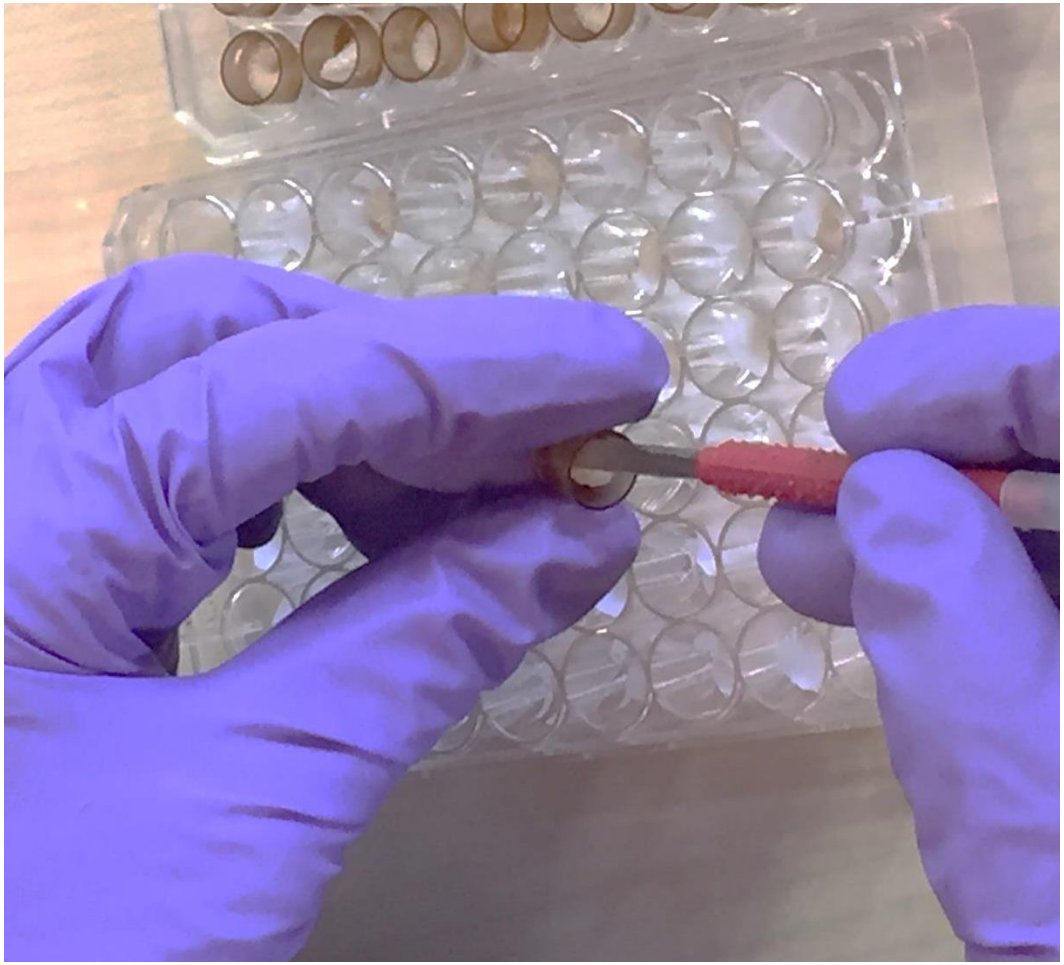
Methodology- Monitoring Larval STCP

Dead larvae have a flat appearance, often sunken into the diet

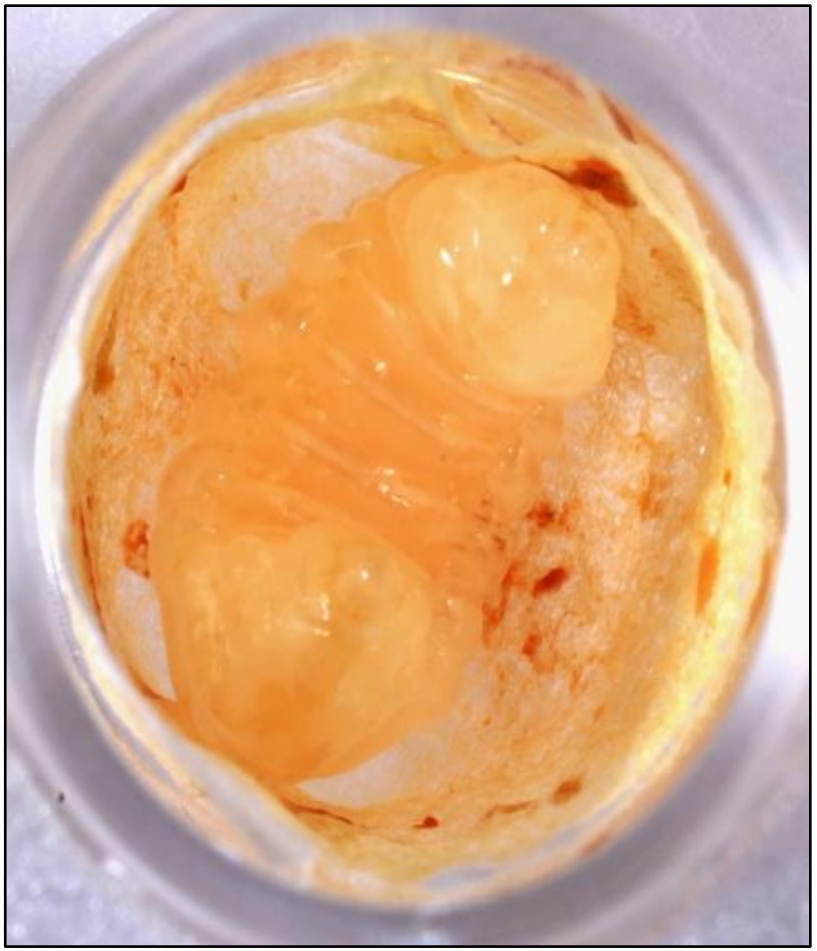
Sometimes have dark spots

Remove entire queen cup from larval STCP

Methodology- Transferring Prepupae



Methodology- Monitoring Pupal STCP



Methodology- Adult Emergence

Methodology Discussion

*Please note that additional methodology instruction will take place on Day 2 of the workshop

-**UF Apiary**: Caging/removing queens, inspecting eggs in frames, frame transport to laboratory

- **Ellis Lab**: Grafting, Method Q&A

Honey bee *in vitro* development

Ring-test goals and discussion

Ring Test (Phase 1)

- Phase 1- Goal: Improve control survival
- Participants and dates
- Endpoints
- Reporting of Data
- Standardization vs Freedom to Operate
  - Create list of what needs to be standardized across all testing laboratories
  - Test questionnaire

"Phase 1" Test

- Phase 1- Goal: Improve control survival
- Participants and dates
- Endpoints
- Reporting of Data
- Standardization vs Freedom to Operate
  - Create list of what needs to be standardized across all testing laboratories
  - Test questionnaire

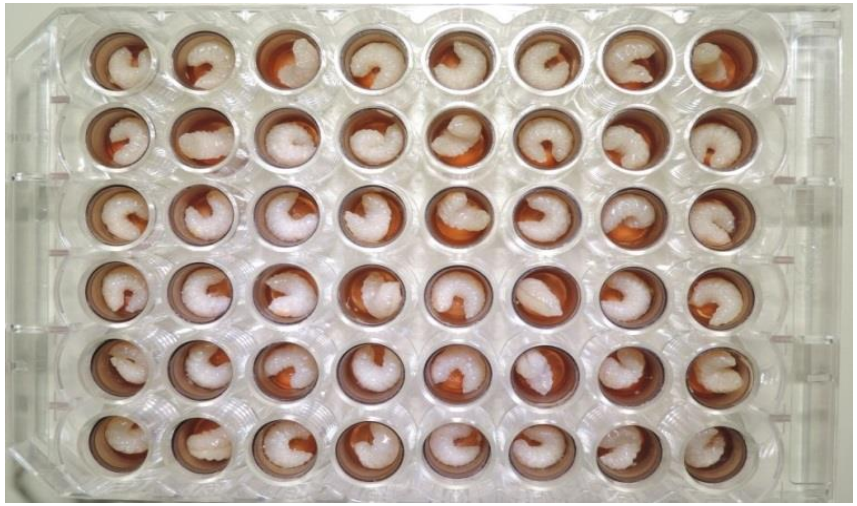
Lab #	Contact Information	Testing Location	Estimate Date of Test
1			
2			
3			
4			
5			
6			

"Phase 1" Test

- Phase 1- Goal: Improve control survival
- Participants and dates
- Endpoints
- Reporting of Data
- Standardization vs Freedom to Operate
  - Create list of what needs to be standardized across all testing laboratories
  - Test questionnaire

“Phase 1” Measures

- Grafting Survival- monitor on day 2 (prior to plate randomization)
- Test Survival



"Phase 1" Test

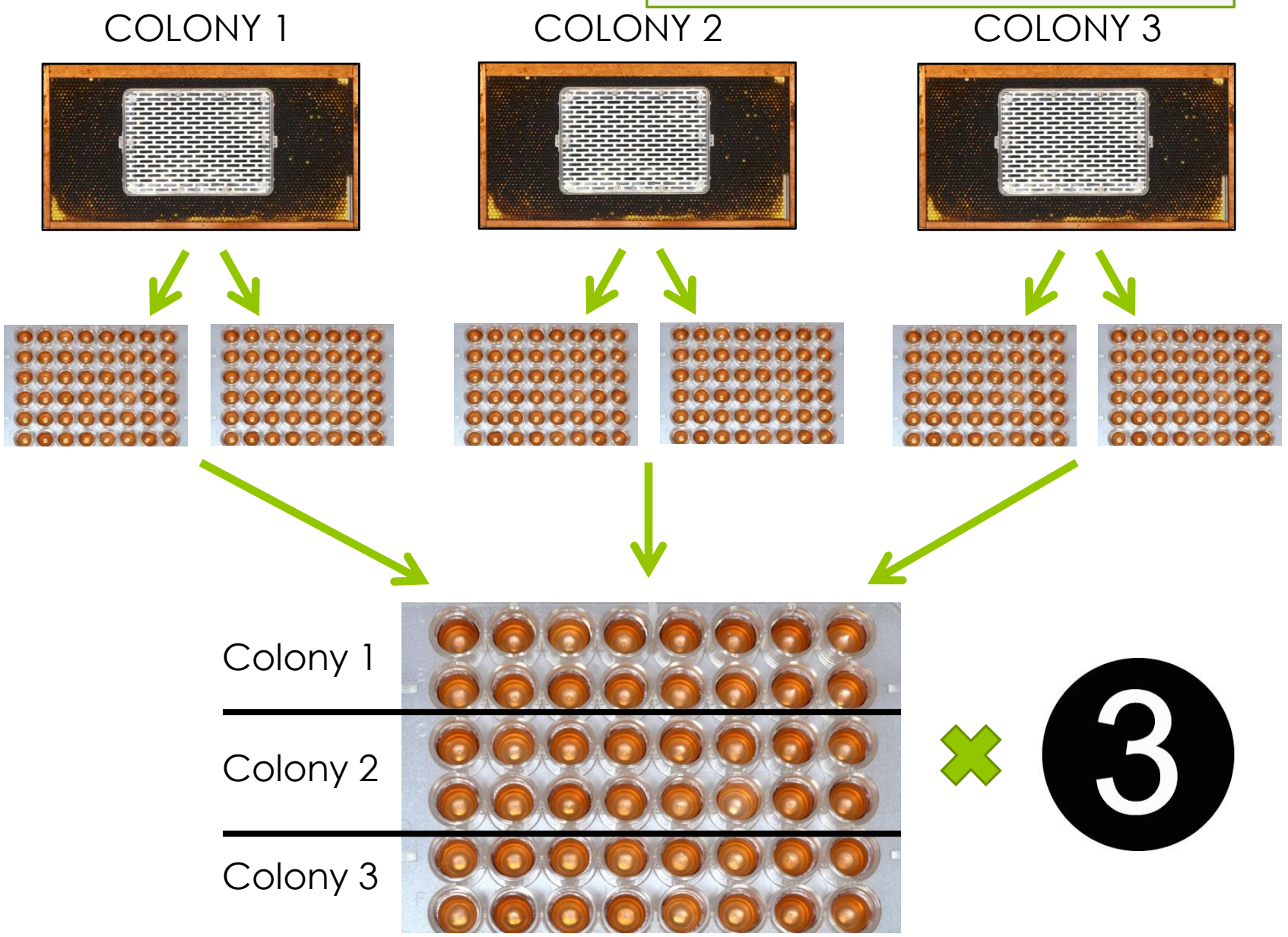
- Phase 1- Goal: Improve control survival
- Participants and dates
- Endpoints
- Reporting of Data
- Standardization vs Freedom to Operate
  - Create list of what needs to be standardized across all testing laboratories
  - Test questionnaire

“Phase 1” Test- Reporting of Data

- All labs participating in the test will submit the data, regardless of outcomes
- Detailed participant questionnaire
  - Example Questions
    1) Royal Jelly Source
    2) Grafting environment (eg. Hood)
    3) Honey bee strain

“Phase 1” Test

- Phase 1- Goal: Improve control survival
- Participants and dates
- Endpoints
- Reporting of Data
- Standardization vs Freedom to Operate
  - Create list of what needs to be standardized across all testing laboratories
  - Test questionnaire


COLONY 1
COLONY 2
COLONY 3
Colony 1
Colony 2
Colony 3
3

"Phase 1" Test Standardization

Standardized

- Rearing Timelines (caging, grafting, transfer, adult emergence)
- Plate Conditions
  - Type of culture plates
  - Type of queen cups
  - Lid on
  - no dental rolls
  - no sterilizing solution
  - Kimwipes in pupal plate
- Incubator Conditions
  - Data logger for temp./RH
  - 2 desiccator chambers
  - 94% RH for Larvae, 75% RH for Pupae
- Sterilization
  - Autoclave, UV hood
  - Face mask
  - Gloves (touching plate, incubator, desiccator)
- Diet
  - Royal Jelly Source (US Only)
    - Stakich Lot # 14020701R stored at 4°C
  - 0.22µm Filtered water (Millipore, manual)
  - Sugars, Yeast standardized
  - Prepare diet, larval plates in hood
  - Measure 1 mL diet to confirm density (1mL diet = ~1.10 g)
  - Place diet in wells of all plates and place in incubator to warm to 35C prior to grafting
- Queen caging
  - Cage queen
  - Uncage queen 24 hrs
  - Graft queen 75 hours after removing queen from excluder
  - Heat pack in box during transportation
- Heating source during grafting
  - Eg. Space heater within hood, coil/hot plate on benchtop

Non-Standardized

- Hood/Benchtop
- Royal Jelly Source
- Grafting Tool
- Pipet
- Type of queen excluder cage
- Method of sterilization
- Weather conditions (Temperature)
- Equipment Manufacturer
- Tools for prepping/mixing diet (do not shake diet)
- Light source
- Disinfectant spray (eg. 10% bleach) for bench tops
- Grafting Environment
- Grafters/# of grafters